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⑤4 Bereitstellung von rep-negativen AAV-Mutanten und hierfür verwendbare Zellen

⑤7 Die Erfindung betrifft die Bereitstellung von rep-negativen AAV-Mutanten und hierfür verwendbare Zellen. Ferner betrifft die Erfindung ein zur Herstellung der Zellen verwendbares Expressionsplasmid.

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Adeno-assoziierte Viren (AAVs) sind einzelsträngige, zu den Parvoviren gehörende DNA-Viren. Zu ihrer Replikation benötigen AAVs Helferviren, insbesondere Adenoviren oder Herpesviren. In Abwesenheit von Helferviren integrieren AAVs in das Wirtszell-Genom, insbesondere an einer spezifischen Stelle von Chromosom 19 bzw. 17.

Auf dem 4,65-kb großen, linearen Genom von humanem AAV-Typ 2 wurden drei virale Funktionen lokalisiert. Die 145 bp langen "inverted repeats" dienen als Replikationsursprung und als cis Signale für Integration und Verpackung. Das cap Gen codiert für drei Strukturproteine und das rep Gen für eine Familie multifunktionaler Regulatorproteine. Die mRNAs für Rep 78 und seine C-terminal gespleißte Version Rep 68 starten am p5 Promotor. Zwei N-terminal verkürzte Versionen von Rep 78 und R 68, nämlich Rep 52 bzw. Rep 40, werden unter der Kontrolle des p19 Promotors exprimiert. Rep Proteine sind für die DNA-Replikation von AAV notwendig. Ferner werden sie für die Genregulation von AAV benötigt.

AAVs unterdrücken die Tumorentwicklung in Tieren. Ferner unterdrücken sie die durch Onkogene bedingte Zelltransformation wie auch die induzierte DNA-Amplifikation. Desweiteren haben AAVs eine antiproliferative Wirksamkeit.

Rep-Proteine von AAV werden für vorstehende Aktivitäten verantwortlich gemacht. Eine Lokalisierung dieser Aktivitäten auf den einzelnen Rep-Proteinen bzw. Domänen davon existiert jedoch nicht. Eine solche wäre aber notwendig, um AAVs therapeutisch einsetzen zu können. Eine Möglichkeit, diese Lokalisierung zu erreichen, liegt in der Untersuchung von AAVs, die Mutationen in den Rep-Proteinen aufweisen. Viele Versuche wurden diesbezüglich durchgeführt. Bisher ist es allerdings nicht gelungen, rep-negative AAV-Mutanten bereitzustellen, die frei von Wildtyp-AAV sind. Solche sind aber für vorstehende Untersuchungen unerlässlich.

Der vorliegenden Erfindung liegt somit die Aufgabe zugrunde, ein Mittel bereitzustellen, mit dem rep-negative AAV-Mutanten ohne vorstehende Nachteile erhalten werden können.

Erfindungsgemäß wird dies durch die Gegenstände in den Patentansprüchen erreicht.

Gegenstand der Erfindung sind somit Zellen, welche die AAV-Rep-Proteine 78 und 52 sowie 40 und/oder 68 stabil exprimieren. Bevorzugt werden Zellen, welche die Rep-Proteine 78, 52 und 40 exprimieren.

Erfindungsgemäße Zellen können in üblicher Weise hergestellt werden. Vorliegend werden Zellen der bekannten Linie HeM1 als Ausgangsmaterial verwendet (vgl. "5th Parvovirus Workshop", Chrystal River, Florida, USA, Nov. 10-14, 1993). Diese Zellen exprimieren die AAV-Rep-Proteine 78 und 52, wobei die Rep 78-Expression unter der Kontrolle von Dexamethason-induzierbarem MMTV-LTR steht. Zellen von HeM1 werden mit einem für Rep 40 codierenden Expressionsplasmid und/oder einem für Rep 68 codierenden bzw. einem Expressionsplasmid transfiziert, das für beide Rep-Proteine codiert. Vorzugsweise wird ein Expressionsplasmid für Rep 40 verwendet, wobei das Expressionsplasmid pCMRep 40 ganz besonders bevorzugt ist. In die-

sem liegt die für Rep 40 codierende DNA zwischen den Schnittstellen NotI und XbaI des bekannten Vektors pKEX-2-XL vor (vgl. Rittner, K.H. et al, Methods Mol. Cell. Biol. 2 (1991), 176-181). pCMRep 40 wurde bei der DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) unter DSM 9491 am 7. Okt. 1994 hinterlegt. Es stellt auch einen Gegenstand der Erfindung dar.

Die durch Transfektion von pCMRep 40 erhaltenen Zellen exprimieren stabil die AAV-Rep-Proteine 78, 52 und 40. Diese Zellen wurden als Zelllinie He 10-1, He 22-2 und He 5-5 bei der DSM unter DSM ACC2193, DSM ACC2192 bzw. DSM ACC21 91 am 28. Sept. 1994 hinterlegt. Sie stellen ebenso einen Gegenstand der Erfindung dar.

Ein weiterer Gegenstand der Erfindung ist ein Verfahren zur Bereitstellung von repnegativen AAV-Mutanten. Ein solches Verfahren umfaßt die folgenden Verfahrensschritte:

- (a) Transfektion erfindungsgemäßer Zellen mit der DNA einer rep-negativen AAV-Mutante,
- (b) Behandlung der transfizierten Zellen von (a) mit einem eine AAV-Replikation ermöglichenden Mittel, insbesondere einem AAV-Helfervirus, und
- (c) Isolierung der in (b) erhaltenen rep-negativen AAV-Mutanten.

Der Verfahrensschritt (a) impliziert auch die Infektion erfindungsgemäßer Zellen mit einer rep-negativen AAV-Mutante. Desweiteren kennt der Fachmann sämtliche zur Durchführung vorstehender Verfahrensschritte notwendigen Techniken. Ergänzend wird auf Maniatis et al, Molecular Cloning: A laboratory manual (1982), Cold Spring Harbor, New York, verwiesen.

Der Ausdruck "DNA einer rep-negativen AAV-Mutante" umfaßt eine für Rep codierende DNA, die jegliche Art von Mutationen tragen kann. Insbesondere kann es sich um Deletionen, Insertionen und/oder Substitutionen von ein oder mehreren Nukleotiden handeln. Auch kann die AAV-DNA eine Deletion des gesamten für Rep codierenden Bereichs aufweisen. Desweiteren kann die Rep codierende DNA teilweise oder ganz durch eine für ein Fremdprotein codierende DNA ersetzt sein. Vorzugsweise ist das Fremdprotein ein für eine Gentherapie verwendbares Protein bzw. Peptid davon. Der Ausdruck "rep-negative AAV-Mutante" impliziert somit auch den Begriff "rep-negativer AAV-Vektor".

Desweiteren umfaßt der Ausdruck "DNA einer rep-negativen AAV-Mutante" auch eine DNA, die neben den vorstehend angegebenen Mutationen weitere Mutationen in anderen Bereichen der AAV-DNA aufweist. Dies können z. B. Mutationen im cap-Gen sein. Für einen solchen Fall ist es gefordert, daß ein exprimierbares AAV-cap-Gen in den erfindungsgemäßen Zellen vorliegt. Dies kann durch das die AAV-Replikation ermöglichende Mittel, z. B. dem AAV-Helfervirus, eingebracht sein. Dem Fachmann sind Verfahren bekannt, ein AAV-cap Gen, z. B. in ein AAV-Helfervirus zu inserieren.

Der Ausdruck "AAV-Helfervirus" umfaßt Viren, die eine Replikation von AAVs ermöglichen. Dies sind insbesondere Adenoviren, wie Adenovirus-2, und Herpesviren.

Mit der vorliegenden Erfindung ist es möglich, rep-negative AAV-Mutanten bereitzustellen. Diese sind frei von Wildtyp-AAV, da Rekombinationsereignisse, wie sie bei transienter Expression von Rep-Proteinen in Zel-

len eintreten, vermieden werden. Die vorliegende Erfindung stellt somit die Basis dar, die den Rep-Proteinen zugeschriebenen Aktivitäten auf einzelne Rep-Proteine bzw. Domänen davon zu beschränken. Damit ist die Möglichkeit gegeben, AAVs für therapeutische Zwecke zu verwenden. Diese finden sich insbesondere im Bereich der Gentherapie. Erfindungsgemäße rep-negative AAV-Mutanten können hierfür verwendbare Gene bzw. Genabschnitte tragen. Diese können insbesondere in dem rep-Gen und/oder cap-Gen liegen. Die vorliegende Erfindung stellt somit einen Durchbruch auf dem Gebiet der Herstellung von für Gentherapien verwendbaren Vektoren dar.

Kurze Beschreibung der Zeichnung

Fig. 1 zeigt eine schematische Darstellung der Rep-codierenden DNA im Expressionsplasmid pCMRep40. Das Start-ATG-Triplett und das Terminationscodon TGA sind angegeben, sie entsprechen denen des Wildtyp-AAV-Genoms. Durch gerichtete Mutagenese ist das Intron (Position: 1907–2227) entfernt, wodurch Rep 40 ohne Spleißen exprimiert werden kann.

Die Erfindung wird durch das Beispiel erläutert.

Beispiel

Bereitstellung einer AAV-Rep-Mutante

He10-1-Zellen werden mit der DNA der bekannten AAV-Rep-Mutante pTAV 2–3 transfiziert. pTAV2-3 weist eine "frameshift"-Mutation an der Position 1045 auf, wodurch alle vier Rep-Proteine inaktiviert sind (vgl. Heilbronn, R., et al, J. Virol. 64 (1990), 3012–3018). Die Zellen werden mit Adenovirus-2 (MOI = 10–20) infiziert. Danach werden sie mit 10^{-6} M Dexamethason induziert.

Nach ca. 30 h werden ein Teil der Zellen geerntet und die Gesamtzell-DNA isoliert. Diese wird mit den Restriktionsenzymen XbaI bzw. DpnI gespalten und in einem Southern-Blot analysiert. Hierzu wird 32 P-markierte AAV-DNA als Hybridisierungsprobe verwendet. Das Restriktionsenzym XbaI schneidet AAV-DNA nicht, ebenso spaltet das Restriktionsenzym DpnI keine in Eukaryoten replizierte DNA. Es wird replizierte pTAV2-3-DNA erhalten.

Desweiteren wird der Überstand der nicht geernteten Zellen abwechselnd eingefroren und aufgetaut sowie einer Ultraschallbehandlung unterzogen. Der Überstand wird auf He10-1-Zellen titriert. Der Nachweis infektiöser AAVs wird durch Hybridisierung mit einer 32 P-markierten Sonde verfolgt, die für rep-negative AAVs spezifisch ist. Es werden infektiöse rep-negative AAV-Partikel nachgewiesen.

Vorstehende Daten zeigen, daß mit den erfindungsgemäßen Zellen rep-negative AAV-Mutanten bereitgestellt werden können.

Patentansprüche

1. Zellen, stabil exprimierend die AAV-Rep-Proteine 78 und 52 sowie 40 und/oder 68.
2. Zellen nach Anspruch 1, dadurch gekennzeichnet, daß sie die AAV-Rep-Proteine Rep 78, 52 und 40 stabil exprimieren.
3. Zellen nach Anspruch 2, nämlich die Zelllinien He 10-1 (DSM ACC2193, He 22-2 (DSM ACC2192) und He 5-5 (DSM ACC2191).

4. Expressionsplasmid, nämlich pCMRep 40 (DSM 9491).

5. Verfahren zur Bereitstellung von rep-negativen AAV-Mutanten, umfassend die folgenden Verfahrensschritte:

- (a) Transfektion der Zellen nach einem der Ansprüche 1-3 mit der DNA einer rep-negativen AAV-Mutante,
- (b) Behandlungen der transfizierten Zellen von (a) mit einem eine AAV-Replikation ermöglichenden Mittel, insbesondere einem AAV-Helfervirus, und
- (c) Isolierung der in (b) erhaltenen rep-negativen AAV-Mutanten.

6. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß die DNA der repnegativen AAV-Mutante von Verfahrensschritt (a) ein oder mehrere Deletionen, Insertionen und/oder Substitutionen im rep Gen aufweist.

7. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß in der DNA der rep-negativen AAV-Mutante von Verfahrensschritt (a) das rep Gen deletiert ist.

8. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß in der DNA der rep-negativen AAV-Mutante von Verfahrensschritt (a) das rep Gen zumindest teilweise durch ein Fremd-Gen ersetzt ist.

9. Verfahren nach einem der Ansprüche 5–8, dadurch gekennzeichnet, daß das AAV-Helfervirus ein Adenovirus oder Herpesvirus ist.

10. Verfahren nach einem der Ansprüche 5–9, dadurch gekennzeichnet, daß die DNA der rep-negativen AAV-Mutante von Verfahrensschritt (a) eine weitere Mutation im cap-Gen aufweist, mit der Maßgabe, daß das die AAV-Replikation ermöglichende Mittel, insbesondere der AAV-Helfervirus, ein exprimierbares cap-Gen enthält.

11. rep-negative AAV-Mutante, erhalten durch das Verfahren nach einem der Ansprüche 5–10.

Hierzu 1 Seite(n) Zeichnungen

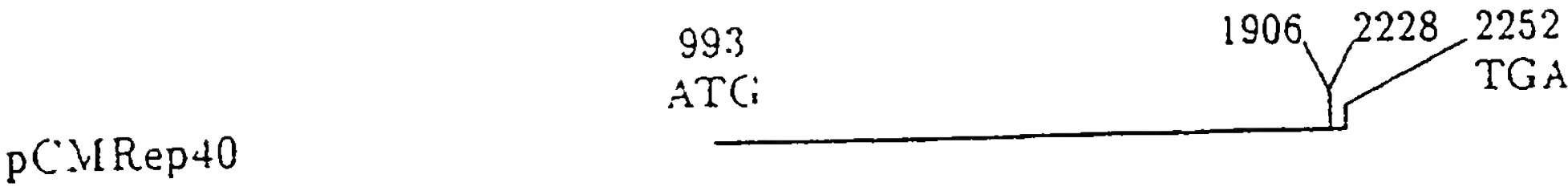


FIG. 1 Schematische Darstellung der Rep-codierenden DNA
im Expressionsplasmid pCMRep40



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(54) Title: ADENO-ASSOCIATED VIRUS - ITS DIAGNOSTIC USE WITH EARLY ABORTION (57) Abstract <p>The present invention relates to a method of detecting a causative agent of the so-called spontaneous early abortion by investigating patients' samples for the presence of adeno-associated virus DNA (AAV DNA), or AAV antigen or antibodies, preferably of the IgM type, directed to AAV. Furthermore, the present invention relates to antibodies suitable for said method.</p>		

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**Adeno-Associated Virus -
Its Diagnostic Use with Early Abortion**

The present invention relates to a method of detecting a causative agent of the so-called spontaneous early abortion by investigating patients' samples for the presence of adeno-associated virus DNA (AAV DNA), or AAV antigen or antibodies, preferably of the IgM type, directed to AAV. Furthermore, the present invention relates to antibodies suitable for said method.

The adeno-associated viruses (AAV) which are human parvoviruses that depend on coinfecting helper viruses for their replication, are thought to be non-pathogenic (Siegl, G. et al. (1985), Intervirology, 23, pp. 61-73; Berns, K.I. et al. (1987), Adv. Virus Res., 32, pp. 243-306) but rather to exhibit tumorsuppressive properties (Rommelaere et al. (1991), J. Virol. Methods, 33, pp. 233-251). The virus may persist in infected individuals, possibly by integration of its DNA into specific chromosomal sites of the host cell genome as seen in cell culture. Recent studies of our laboratories have demonstrated that AAV is able to induce differentiation in a variety of cells of human and mouse origin (Klein-Bauernschmitt et al. (1992), J. Virol., 66, pp. 4191-4200) including embryonic stem cells. In the course of looking for putative targets of AAV infection, we analysed material from spontaneous abortion for the presence of AAV DNA using for example the polymerase chain reaction (PCR), the Southern blotting technique and the in situ hybridization technique. Additionally, we analysed serum samples from women with miscarriage and from other diseased or healthy women for the presence of antibodies to AAV using serological standard techniques such as enzyme linked immunosorbent assay (ELISA), fluorescenceimmuno assay (FIA), radio-immuno assay (RIA) or immunofluorescence assay (IFA).

Surprisingly, we found a significant correlation of both detectable AAV DNA in samples of abortion material and detectable IgM antibodies directed to AAV with the early abortion occurring during the first trimester of pregnancy.

Disclosure of the invention

Accordingly, the present invention relates to a method of detecting the causative agent of spontaneous abortion comprising the steps of

5 a) hybridizing a probe for an AAV polynucleotide to nucleic acids of a sample of abortion material under conditions which allow the formation of a heteroduplex between an AAV nucleic acid and the probe, and

10 b) detecting a polynucleotide duplex which contains the probe.

15 In a preferred embodiment of the present invention the method as mentioned above is a polymerase chain reaction (PCR), Southern blotting or in situ hybridization technique.

20 In another preferred embodiment of the present invention a hybridization technique is applied as described above, wherein one or more nucleic acid probes are used which are selected from the group consisting of the primers pan1, pan3, nest1 and nest2. In Figure 1 a schematic drawing of these primers, relative to the genome of the AAV type 2 (AAV-2) and the nucleotide sequences of the primers is presented.

25 The present invention further relates to a method of detecting the causative agent of spontaneous abortion comprising the steps of

a) incubating a probe antibody directed to an AAV antigen with a sample of abortion material under conditions which allow the formation of an antigen-antibody complex, and

30 b) detecting the antigen-antibody complex containing the probe antibody.

- 3 -

In step (a) one or more probe antibodies can be used. These antibodies can be directed to e.g. an AAV capsid or a single protein thereof, particularly VP1, VP2 or VP3. Examples of these antibodies are the following monoclonals:

5

A1; deposited at DSM under DSM ACC2195 on Oct. 13, 1994

A69; deposited at DSM under DSM ACC2196 on Oct. 13, 1994

B1; deposited at DSM under DSM ACC2197 on Oct. 13, 1994

A20; deposited at DSM under DSM ACC2194 on Oct. 13, 1994

10

(see Table 1).

The antibodies as mentioned above are subject matter of the present invention.

15

In a preferred embodiment of the present invention the method of antigen detection as mentioned above is an enzyme linked immunosorbent assay (ELISA), a radioimmuno assay (RIA), a fluorescence immuno assay (FIA) or an immunofluorescence assay (IFA).

20

An example of the ELISA comprises the following steps:

(a) providing a substrate carrying the monoclonal antibody A 20,

(b) contacting the substrate of (a) with a sample of abortion material to get an antigen-antibody complex,

25

(c) contacting the complex of (b) with a polyclonal anti-AAV capsid antibody to get an antibody-antigen-antibody complex,

(d) contacting the complex of (c) with an enzyme-labelled antibody directed to the polyclonal antibody of (c) to get a labelled complex of (c), and

30

(e) contacting the complex of (d) with an enzyme-label-indicator to indicate the presence of said complex.

- 4 -

It is clear that the term "sample of abortion material" is only an example of materials which contain AAV capsids or parts thereof. Another examples are cells expressing recombinant AAV capsids or parts thereof.

5 The present invention, i.e. the antibodies alone or in combination with the AAV antigen detection method, is suitable to detect AAV capsids and/or parts thereof in any material.

10 Furthermore, the present invention relates to a method of detecting the causative agent of spontaneous abortion comprising the steps of

- 15 a) incubating a sample containing AAV or an antigenic part thereof with a sample suspected of containing anti-AAV antibodies under conditions which allow the formation of an antibody-antigen complex, preferably only containing antibodies of the IgM type, and
- b) detecting an antibody-antigen complex, preferably IgM antibody-antigen complex, containing the probe antigen.

20 In step (a) the term "sample containing AAV or an antigenic part thereof" refers to AAV capsid proteins, particularly VP1, VP2 and/or VP3, preferably.

25 In another preferred embodiment of the present invention the method of detection of AAV specific antibodies, particularly IgM antibodies, is an ELISA, a RIA, a FIA or an IFA.

An example of the ELISA comprises the following steps:

- 30 (a) providing a substrate carrying an anti-human IgM antibody,
(b) contacting the substrate of (a) with a patient's bodyfluid to get an antibody-antibody complex,

- 5 -

- (c) contacting the complex of (b) with recombinant VP1, VP2 and/or VP3 to get a VP-antibody-antibody complex,
- (d) contacting the complex of (c) with an anti-VP-antibody to get an anti-VP-antibody-VP-antibody-antibody complex,
- 5 (e) contacting the complex of (d) with an enzyme-labelled antibody directed to the anti-VP-antibody of (d) to get a labelled complex of (d), and
- (f) contacting the complex of (e) with an enzyme-label-indicator to indicate the presence of said complex.

10

It is evident that persisting anti-AAV IgM/IgG titers in serum are associated with predisposition to early abortions. Thus, the present invention can also be used for effective risk factor screening, development of methods for prevention of pregnancy failure, and information of patients about the risks of pregnancy failure.

15

Furthermore, the present invention relates to a kit for detecting the causative agent of spontaneous abortion by hybridization as described above, comprising a probe for an AAV polynucleotide in a suitable container.

20

The present invention further relates to a kit for detecting the causative agent of spontaneous abortion by immunological antigen detection as described above, comprising a probe antibody directed against an AAV antigen in a suitable container.

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The present invention further relates to a kit for detecting the causative agent of spontaneous abortion by immunological antibody detection as described above, comprising AAV or an antigenic part thereof in a suitable container.

30

Modes for carrying out the invention:

The art is rich in methods available to the man of the art in recombinant

- 6 -

nucleic acid technology, microbiology and immunobiology for carrying out the present invention. Detailed descriptions of all of these techniques will be found in the relevant literature. See for example Maniatis, Fritsch & Sambrook: Molecular Cloning: A Laboratory Manual (1989); DNA Cloning, Vol. I and II (D.N. Glover ed., 1985); Oligonucleotide Synthesis (M.J. Gait ed., 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds., 1984); Animal Cell Culture (R.I. Freshney ed., 1986); J.D. Watson, M. Gilm-an, J. Witkowski, M. Zoller: Recombinant DNA, Second Edition (1992); Immunochemical Methods in Cell and Molecular Biology (Academic Press, London, 1987); Protein Purification: Principles and Practice, Second Edition (Springer Verlag, N.Y.); Handbook of Experimental Immunology, Vol. I-IV (D.M. Weir and C.C. Blackwell eds., 1986); Immunoassay: A Practical Guide (D.W. Chan and M.T. Perlstein eds., 1987); ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects (D.M. Kemeny and S.J. Challacombe eds., 1988); Principles and Practice of Immunoassay (C.P. Price and D.J. Newman eds., 1991).

More detailed information on specific methodological aspects of AAV, such as cell culture, virus growth, virus purification, isolation of proteins, can be found in the relevant literature, e.g. Handbook of Parvoviruses, Vol. I and II CRC Press, Boca Raton, Florida, Ed. P. Tijssen; Ruffing, M. et al. (1992), J. Virol., 66, pp. 6922-6930.

All reagents such as antigens, antibodies, probe antigens, probe antibodies, nucleic acid probes, primers and auxilliary reagents necessary to perform an immunoassay or a hybridization assay, possibly using amplification techniques for improved sensityity may be filled into suitable containers or coated to any solid phase such as plastic, glass and cells, and packaged into kits together with instructions for conducting the test.

The present invention is exemplified by the following examples.

- 7 -

Example 1:

Detection by Polymerase-Chain-Reaction (PCR) - analysis of AAV DNA in biological, e.g., curettage material of spontaneous abortion.

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The primers used in PCR (pan1, pan3) and nested PCR (nest1, nest2), respectively, were designed to hybridize to sequences of AAV-2 and AAV-5 DNA by allowing mismatches not leading to amplification of other (e.g. cellular) DNA sequences. The amplified products are distinguishable by Southern blot experiments. The primers were prepared according to standard procedures.

The primers were designed displaying mismatches (underlined) as shown below:

15 - - - - - AACTGGACCAATGAAAACTTTCC - - - - - pan1

1386 TCGGTAAACTGGACCAATGAGAACTTTCCCTTCAAC AAV-2

130 TCGGTAAACTGGACCAATGAAAACTTTCCCTTCAAC AAV-5

20

AAAAAGTCTTTGACTTCCTGCTT pan3

1729 AAAAAGTCTTTGACTTCCTGCTT AAV-2

25

472 AAAAAGTCCTTGACTTCCTGCTT AAV-5

30

DNA prepared from histological sections (5 μ m, of fresh or fixed, paraffin-embedded, deparaffinated material [Methods as described by D.H. Wright and M.M. Manos in "PCR Protocols, A Guide to Methods and Applications", edited by M.A. Innis, D.H. Gelfand, J.J. Snoisky and T.L. White, Chapter 19, pp. 153-158; Academic Press, New York, 1990] were analysed by PCR using

- 8 -

the primers pan1 and pan3 combined, followed in AAV positive cases by a repetition of the PCR (to confirm specificity) using the (internal) primers nest1 and nest2 (see Figure), respectively. PCRs were performed for 40 cycles (one cycle = 92 °C, 1 min; 62 °C, 4 min; 92 °C, 15 sec) (van den Brule et al., (1989) J. Med. Virol., 29, pp. 20-27). Amplified products were characterized by electrophoretic separation (2 % agarose gel) and blotting onto a nylon membrane (Gene Screen, NEN, Dupont, Dreieich, Germany) followed by hybridization at high stringency with ³²P-labelled probes (labelled using the Megaprime™ DNA Labelling System, Amersham, UK) of AAV-2 (pTAV2 [Heilbronn et al. (1990), J. Virol., 64, pp. 3012-3018]) or of AAV-5. This probe was cloned from DNA from purified AAV-5 virions, propagated with adenovirus type 12 and purified as described in de La Maza and Carter (1980), J. Virol., 33. pp. 1129-1137 and in Rose (1974) Parvovirus Reproduction, pp. 1-61; In: H. Fraenkel-Conrat and R.R. Wagner, eds., Comprehensive Virology, Plenum Press, New York.

Example 2:

Detection by Southern Blotting analysis of AAV DNA in fresh curettage material

Genomic DNA was isolated using standard procedures with minor modification (Laird et al. (1991), Nucl. Acids Res., Vol. 19, pp. 4293-4294) and digested with restriction enzymes allowing analysis of characteristic restriction sites within the AAV genome. After separation through 0,8 % agarose gels, DNA fragments were blotted onto Nylon membranes (Gene Screen) and hybridized AAV-2 DNA (pTAV2, see Example 1) or specific AAV-5 DNA (see Table 2) labelled by random priming with [α -³²P] dCTP (Amersham, Braunschweig, Germany).

- 9 -

Example 3:

Detection of AAV DNA by in situ hybridization in sections of biopsy material,
e.g. curettage from spontaneous abortion

5 In situ hybridization was performed as described (Tobiasch et al. (1992)
Differentiation, 50, pp. 163-178), however, with the modification that AAV-2
DNA was detected by RNA-DNA hybridization. After DNase treatment, the
probes were subjected to limited alkaline hydrolysis. Upon linearisation of the
10 plasmid pTAV2 (Heilbronn et al. (1990), see above) with EcoRV, riboprobes
were obtained and labelled with [³⁵S]-UTP by in vitro transcription with T7
RNA polymerase (method as described in Boehringer Mannheim Procedure
supplied with the "SP6/17 Transcription Kit"). Prior to hybridization, both
probe and target DNA were denatured (93 °C, 10 min). For in situ hybridiza-
15 tion with [³²P-]-UTP labelled probes, the protocol was as described in Dürst
et al. (1992) Virology, 189, pp. 132-140.

Example 4

20 Provision of antibodies directed to AAV capsid proteins

In order to generate monoclonal antibodies directed to AAV capsid proteins
two BALB/c mice were injected subcutaneously (s.c.) with 150 µl of a
25 mixture of gel purified recombinant capsid proteins in PBS containing 100 µg
each of VP1, VP2 and VP3, mixed with an equal volume of complete
Freund's adjuvant. After four weeks the mice were boosted s.c. with 25 µg
of purified UV-inactivated AAV-2 in 50 µl PBS and 50 µl incomplete
Freund's adjuvant. After four weeks the mice were injected intraperitoneally
30 (i.p.) each with 10 µg of UV-inactivated AAV-2 in 100 µl PBS. Three days
later one mouse was killed and the spleen cells were fused with X63/Ag8
cells according to standard procedures (Harlow, E. and Lane, D. (1988), Cold

- 10 -

Spring Harbor Laboratory, Antibodies, A laboratory manual). Resultant hybridoma culture supernatants were screened by Western blotting, immunofluorescence and ELISA. The second mouse was immunized six months later with 100 μ g of purified VP3 in PBS (i.p.) and monoclonal antibodies were prepared as described above.

Example 5:

ELISA for the detection of IgG antibodies directed to AAV

96-well microtiterplates (Nunc, Denmark) were coated with 50 μ l CsCl-gradient purified AAV 2 (dilution 1:1000 in 0,05 M carbonate-buffer pH 9,6) or with 50 μ l recombinant AAV 2 capsid proteins VP1-3 (1:8000 in 0,05 M carbonate-buffer) and incubated over night at RT. Plates were washed twice (washing buffer: PBS, 0,05 % Tween 20) and human sera were added (50 μ l/well, dilutions 1:25 to 1:800, dilution buffer: PBS, 2 % BSA, 0,05 % Tween 20) and incubated for 1 h at 37 °C in a wet chamber. After washing plates were incubated with 50 μ l/well peroxylase conjugated monkey anti-human IgG antibody (1:2000) for 45 minutes at 37 °C in a wet chamber. Plates were washed four times and 50 μ l substrate solution (5 mg OPD in 25 ml 0,1 M citratebuffer pH 5,0 + 10 μ l H₂O₂ 35 %) was added. Plates were stored for 10-15 minutes in the dark and the reaction was stopped with 50 μ l 1N H₂SO₄/well. Extinctions were measured at 492 nm in a Titertek photometer. Background signal was determined by measuring the extinction without adding human sera and was subtracted on every well (background signal extinction ranged from 0,035 to 0,05).

Example 6:

ELISA for the detection of IgM antibodies directed to AAV

- 11 -

Version A

Plates were coated as described in Example 4. Human sera were added after they had been treated according to the following absorption protocol in order to eliminate remaining IgG-antibodies : 20 μl absorption reagent (FREKA-Fluor, Fresenius, Germany) were diluted with 25 μl PBS and 5 μl of human serum was added. Absorption was performed for at least 15 minutes at RT, and subsequently sera were tested at dilutions from 1:100 to 1:800. Incubation was performed for 1 h at 37 °C in a wet chamber and after washing 50 μl /well peroxidase conjugated goat anti human IgM antibody (1:2000 in PBS/2 % BSA/0,05 % TWEEN 20) were added. Plates were incubated for 45 minutes at 37 °C and washed four times. The OPD reaction and photometric evaluation were performed as described in Example 5.

Version B **μ -capture ELISA****Plate Coating**

Rabbit anti-human IgM antibody (DAKO) was first denatured at a protein concentration of 600 $\mu\text{g/ml}$, incubating for 30 min at RT in 50mM glycine/HCl pH 2,5 containing 100 mM NaCl then neutralized with 1 M Tris base. The denatured antibody was then desalted by passing the solution over a Sephadex PD 10 column equilibrated in the coating solution (10mM Tris/HCl pH 8,5 containing 100 mM NaCl). The sample was eluted from the column in the same buffer. The solution was adjusted to a protein concentration of 6 $\mu\text{g/ml}$ by dilution in coating buffer and 200 μl added to each well on a polystyrene microtiter plate (NUNC immuno flat-bottomed well). The plate was incubated at 37°C for 24 h in a humid atmosphere, contents decanted and wells washed 4 times with 250 μl /well of Tris-buffered saline (TBS) (0,02 M Tris/HCl pH 7,4, 0,15 M NaCl) containing 0,05 % Tween 20 (wash buffer). The wells were then blocked with TBS containing 1 % Tween 20 and 5 % Sucrose (blocking solution) by incubating at 4°C followed by 2 washings in wash buffer (TBS containing 0,05% Tween 20).

- 12 -

Assay

The second step in the ELISA involved contacting patients' sera with the antibody-coated plate. During incubation, IgM was immunologically bound to the solid-phase antibody. After removal of the unbound material and washing of the microtiter plates, the plates were incubated with purified recombinant AAV nucleocapsid proteins VP1, VP2 and VP3. After removal of the unbound material and washing of the microtiter plates, complexes of human IgM antibody-VP complexes were detected by incubation with the A1, A69 and B1 antibodies. Unbound monoclonal antibodies were removed by aspiration and the plates were washed. The bound monoclonal antibodies were detected by incubating the plates with goat anti-mouse immunoglobulin antibodies conjugated to horseradish peroxidase (HRP). Following removal of unbound conjugate by washing, a solution containing H_2O_2 3.3', 5.5' tetramethylbenzidine (TMB) was added. Reactions were stopped after a suitable interval by addition of sulfuric acid. The cutoff value of the ELISA was calculated as the average optical density of five negative samples plus 3 standard deviations (to correct for any aspecific binding). Samples giving absorbance values higher than the cutoff were considered positive.

Specifically, the anti-human IgM on the plate was reacted with serum by adding 100 μ l of serum samples diluted 1:200 in TBS containing 10 mg/ml bovine serum albumin, and incubating the serum-containing wells for 1 h at room temperature. After incubation, the serum samples were removed by aspiration and the wells were washed 5 times with washing solution (TBS + 0,05% Tween 20). Aliquots of 100 μ l of the VP1, VP2 and VP3 antigen mixture (conc of 10-10 nM VP1, VP2 and VP3) were added to each well and the plates were incubated at room temperature at least 2 h, followed by removal of excess probe by aspiration and 5 washes with TBS + 0,05 % Tween 20. Bound VP1, VP2 and VP3 was detected by addition of 100 μ l of a mixture of hybridoma supernatants from A1, A69 and B1 monoclonal antibodies producing hybridomas (antibody conc 1-10 nM), followed by 5 standard washes of the plates with TBS + 0,05% Tween 20. Monoclonal

- 13 -

antibody binding was detected by addition of 200 μ l of 1 1/2000 dilution of sheep anti-mouse IgG horseradish peroxidase-conjugated antibody (Dako, Hamburg/Germany) and incubated for 1,5 h at room temperature, followed by 5 standard washings of the plate. Enzyme activity was revealed by addition of 100 μ l of a solution of TMB (Serex, Maywood, N.J./USA). The plate was incubated until the desired color development was reached and terminated by addition of 50 μ l 2N sulfuric acid. Optical densities (OD_{450}) of negative and positive control sera as well as samples were determined. The cutoff value as calculated from five negative sera was $OD_{450} = 0,40$.

Example 7:

ELISA for the detection of AAV capsids

Plate Coating

100 μ l of the A20 antibody (see above) equilibrated in coating buffer solution (50 mM $NaHCO_3$ pH 9.6 and adjusted to a protein concentration of 1,5 ng/ml) was added to each well on a polystyrene microtiter plate (NUNC immuno flat-bottomed well). The plate was incubated at 4 °C for 24 h, contents decanted and wells, washed 5 times with 250 μ l/well of phosphate-buffered saline (PBS) (wash buffer). The wells were blocked with 260 μ l of 3 % BSA in PBS (blocking solution) by incubating at least 30 minutes at room temperature followed by 6 washings in wash buffer.

Assay

A standard curve within the range of 10 - 10 000 capsids/ml was prepared by diluting AAV capsids in standard dilution solution containing PBS.

Unknown samples were diluted as appropriate in diluent solution and 100 μ l added to the test wells. When tissue culture supernatants were to be assayed, 100 μ l of a 1:10 to 1:10⁸ dilution was to be added to the test well.

- 14 -

The plate was incubated for 3 h at room temperature. The plate was washed 5 times in wash buffer and 100 μl rabbit anti-AAV-polyclonal antiserum at a dilution of 1/1000 in 3 % BSA in PBS added to each well. The plate was incubated at room temperature for 2 h as previously and then washed 5 times in PBS Tween. AAV capsid was detected by addition of 100 μl of a 1/2000 dilution of a goat anti-rabbit IgG myeloperoxidase-conjugated antibody prepared in antibody diluent and incubated for 1 h at room temperature followed by 5 standards washes of the plate. Enzyme activity was revealed by addition of 100 μl of a 0,1 mg/ml solution of tetramethylbenzidine (TMB) prepared in 0,1 M Na-acetate buffer pH 6 to each well. The plate was incubated at room temperature until the desired color development was reached, longer incubation periods being necessary to detect lower concentration ranges, i.e. standards less than 10 capsids/ml. The concentration of unknown samples was determined by comparison of their optical density to the standard curve.

Example 8:

Detection of AAV-DNA in curettage materil of spontaneous absorption

A total of 50 samples of curettage material of spontaneous absorption were analysed for the presence of AAV DNA either by PCR or Southern Blotting or both. 41 samples were from abortions in the first and 9 samples from abortions in the second and third trimester of pregnancy.

Among the 41 samples taken during the first trimester of pregnancy, 14 consisted of fresh material that could be tested by Southern Blotting, by which method 9 samples were shown to be positive. All other samples tested were sections from paraffin-embedded tissues, that were analysed by PCR. Among these, 30 samples were from abortions in the first trimester of pregnancy, of which 12 samples were shown to be positive for AAV DNA. All of the 9 samples from the second or third trimester of pregnancy were negative by

- 15 -

PCR.

Thus, in 21 of 41 samples, i.e. 50 % of spontaneous abortions in the first trimester of pregnancy AAV specific DNA sequences could be detected, whereas 9 spontaneous abortions in the second or third trimester were negative (see Table 3).

Example 9:

10 A total serum of 148 serum samples drawn from healthy probands, diseased patients with various syndromes being unrelated to abortion, and pregnant women with spontaneous abortion during the first trimester of pregnancy were tested for antibodies directed to AAV.

15 The results obtained are displayed in Table 4. Generally, the prevalence of specific IgG antibodies was quite high, between 62 and 100 % in the different groups of probands/patients. However, specific IgM antibodies were shown to be significantly correlated with "pregnancy problems".

Table 1

Term	Subtype	Epitope	Western Blotting	Immuno- Precipitation	Immuno- Fluorescence	Characteristics
A1	IgG2a	between aa 1 - 104	+ specific recognition of VP1	+	+	recognition of monomeric and oligomeric VP1
A69	IgG1	between aa 105 - 136	+ specific recognition of VP1 and VP2	++	++	recognition of monomeric and oligomeric VP1 and VP2
B1	IgG1	between aa 136 - 669	++ recognition of VP1, VP2 and VP3	++	++	recognition of monomeric and oligomeric VP1, VP2 and VP3
A20	IgG3	presumable conformation	- (negativ)	+++	++	preferable recog- nition of AAV capsid, no reaction with recombinant monomeric capsid protein

aa: amino acid6

- 17 -

1 Table 2

5 388 bp part of BamH1b fragment of AAV5

10 487 TCAATCAGGTGCCGGTGACTCACGAGTTTAAAGTTCCCAGGGAATTGGCGGGAAGTAAAG
AGTTAGTCCACGGCCACTGAGTGCTCAAATTTCAAGGGTCCCTTAACCGCCCTTGATTTC 546

547 GGGCGGAGAAATCTCTAAACGCCCCACTGGGTGACGTCACCAATACTAGCTATAAAAGTC
CCCGCCTCTTTAGAGATTTTGCGGGTGACCCACTGCAGTGGTTATGATCGATATTTTCAG 606

15 607 TGGAGAAGCGGGCCAGGCTCTCATTTGTTCCCGAGACGCCTCGCAGTTCAGACGTGACTG
ACCTCTTCGCCCCGGTCCGAGAGTAAACAAGGGCTCTGCGGAGCGTCAAGTCTGCACTGAC 666

20 667 TTGATCCCGCTCCTCTGCGACCGCTCAATTGGAATTCAAGGTATGATTGCAAATGTGACT
AACTAGGGCGAGGAGACGCTGGCGAGTTAACCTTAAGTTCCATACTAACGTTTACACTGA 726

25 727 ATCATGCTCAATTTGACAACATTTCTAACAAATGTGATGAATGTGAATATTTGAATCGGG
TAGTACGAGTTAAACTGTTGTAAAGATTGTTTACACTACTTACACTTATAAACTTAGCCC 786

30 787 GCAAAAATGGATGTATCTGTCACAATGTAACCTCACTGTCAAATTTGTCATGGGATTCCCC
CGTTTTTACCTACATAGACAGTGTTACATTGAGTGACAGTTTAAACAGTACCCTAAGGGG 846

35 847 CCTGGGAAAAGGAAAAGTTCAGATTT
GGACCCTTTTCCTTTTGAACAGTCTAAA 874

Table 3

Prevalence of AAV DNA in curettage materials

Diagnosis / Pathology	Detection of AAV DNA by (number AAV positive / number analysed)		
	PCR	Southern Blotting	Total
spontaneous abortion (1st trimester of pregnancy)	12/30	9/14	21/41*
abortion 2nd trimester	0/3	n.d.	0/3
abortion 3rd trimester or placenta post partum	0/6	n.d.	0/6

n.d. = not done;

* = 3 samples positive with PCR were tested by Southern blotting analysis

Table 4

Serum Antibodies to AAV Diagnosis	n	IgG- IgM-	IgG+ IgM-	IgG- IgM+	IgG+ IgM+	IgG+ n	%	IgM+ n	%
<i>Controls (all)</i>	58	8	45	2	3	48	83	5	8,6
Employees	32	4	24	2	2	26	81	4	12,5
Patients *)	26	4	21	0	1	22	85	1	4
<i>breast (all)</i>	38	1	32	0	5	37	97	5	13,2
mammary dystrophy	19	1	13	0	5	18	75	5	26
breast cancer	19	0	19	0	0	19	100	0	0
<i>cervix uteri (all)</i>	26	2	17	4	3	20	77	7	27
normal (or metaplasia)	3	1	2	0	0	2	67	0	0
CIN / CIS	22	1	14	4	3	17	77	7	32
cancer	1	0	1	0	0	1	100	0	0
<i>pregnancy problems (all)</i>	26	6	12	2	6	18	69	8	31
Extra uterine	2	0	2	0	0	2	100	0	0
chromosomal aberrations	3	0	2	0	1	3	100	1	33
abortion (1st trimester) of unclear etiology	21	6	8	2	5	13	62	8	38

*) with uterus myoma, or normal pregnancy, hysterectomy (normal)

- 20 -

CLAIMS:

1. A method of detecting the causative agent of spontaneous abortion comprising the steps of
 - (a) hybridizing a probe for an AAV polynucleotide to nucleic acids of a sample of abortion material under conditions which allow the formation of a heteroduplex between an AAV nucleic acid and the probe, and
 - (b) detecting a polynucleotide duplex which contains the probe.
2. The method according to claim 1, which is a PCR, Southern blotting or an in situ hybridization technique.
3. The method according to claim 1, wherein one or more probes are used which are selected from the group consisting of the primers pan1, pan3, nest1 and nest2.
4. A method of detecting the causative agent of spontaneous abortion comprising the steps of
 - (a) incubating a probe antibody directed to an AAV antigen with a sample of abortion material under conditions which allow the formation of an antigen-antibody complex, and
 - (b) detecting the antigen-antibody complex containing the probe antibody.
5. The method according to claim 4, wherein the probe antibody is A1 (DSM

- 21 -

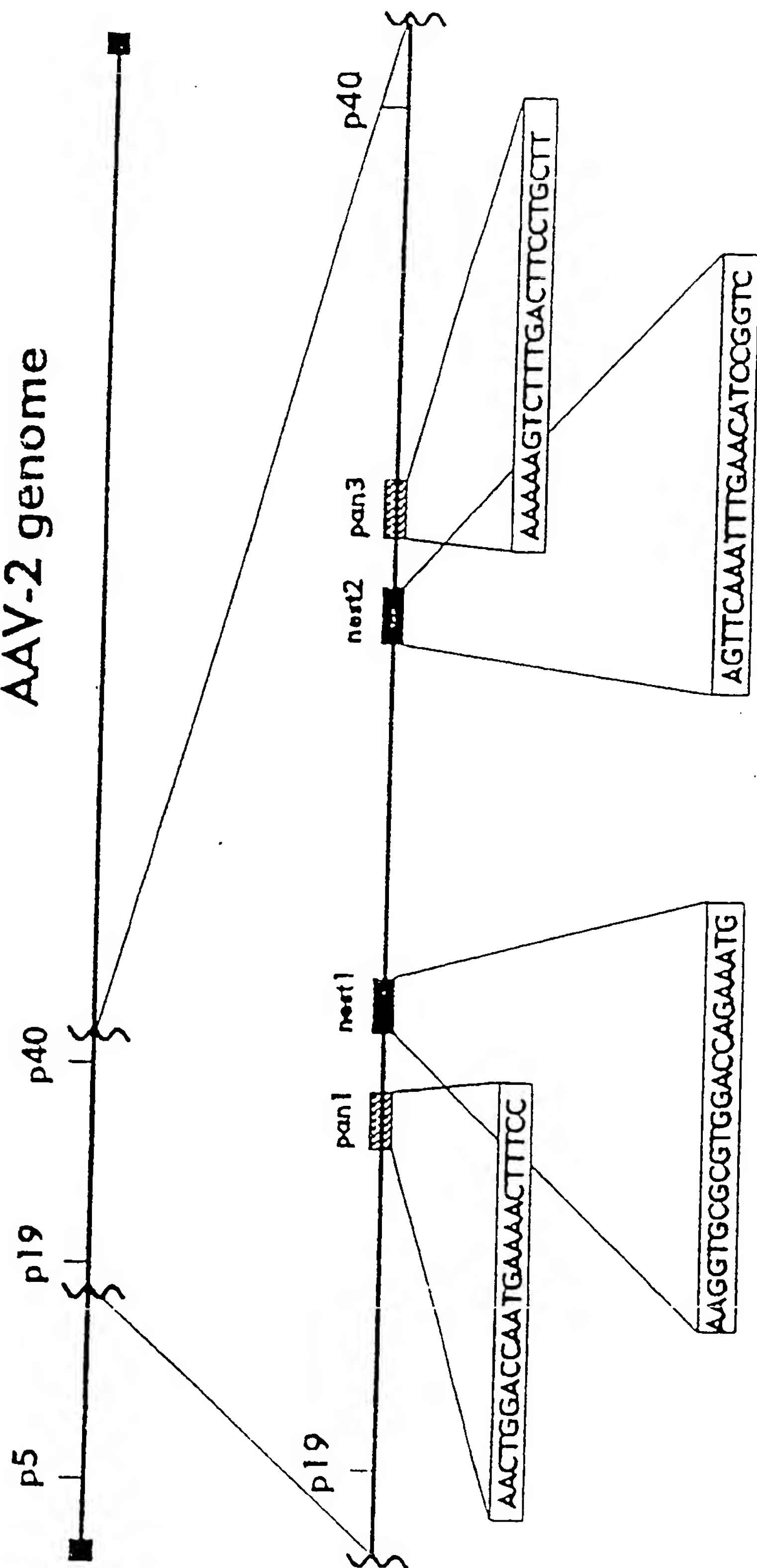
ACC2195, deposited on 13. 10. 1994), A20 (DSM ACC2194, deposited on 13. 10. 1994), A69 (DSM ACC2196, deposited on 13. 10. 1994) and/or B1 (DSM ACC2197, deposited on 13. 10. 1994).

6. The method according to claim 4 or 5, which is an ELISA, a RIA, a FIA or an IFA.
7. A method of detecting the causative agent of spontaneous abortion comprising the steps of
 - (a) incubating a sample containing AAV or an antigenic part thereof with a sample suspected of containing anti-AAV antibodies under conditions which allow the formation of an antibody-antigen complex, and
 - (b) detecting the antibody-antigen complex, containing the probe antigen.
8. The method according to claim 7, wherein the antigenic part of AAV is VP1, VP2 or VP3.
9. The method according to claim 7 or 8, wherein the antibody in the antibody-antigen complex is of the IgM type.
10. The method according to one of claim 7 to 9, which is an ELISA, a RIA, a FIA or an IFA.
11. A kit for performing the method according to claim 1, comprising a probe for an AAV polynucleotide in a suitable container.
12. A kit for performing the method according to claim 4, comprising a probe antibody directed to an AAV antigen in a suitable container.

- 22 -

13. The kit according to claim 12, wherein the probe antibody is A1 (DSM ACC2195, deposited on 13.10.1994), A20 (DSM ACC2194, deposited on 13.10.1994), A69 (DSM ACC2196, deposited on 13.10.1994 and/or B1 (DSM ACC2197, deposited on 13.10.1994).
14. A kit for performing the method according to claim 7, comprising AAV or an antigenic part thereof in a suitable container.
15. The kit according to claim 14, wherein the antigenic part of AAV is VP1, VP2 and/or VP3.
16. Antibody directed to an AAV antigen.
17. Antibody according to claim 16, wherein the antibody is directed to an AAV capsid or a protein thereof.
18. Antibody according to claim 17, wherein the antibody is A1 (DSM ACC2195, deposited on 13.10.1994).
19. Antibody according to claim 17, wherein the antibody is A20 (DSM ACC 2194, deposited on 13.10.1994).
20. Antibody according to claim 17, wherein the antibody is A69 (DSM ACC2196, deposited on 13.10.1994).
21. Antibody according to claim 17, wherein the antibody is B1 (DSM ACC2197, deposited on 13.10.1994).

AAV-2 genome



A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/70 G01N33/569 C07K14/015 C07K16/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OBSTETRICS AND GYNECOLOGY, vol.81, no.3, March 1993, NY US pages 402 - 408 ROGERS B B et al 'DETECTION OF HUMAN PARVOVIRUS B19 IN EARLY SPONTANEOUS ABORTUSES USING SEROLOGY HISTOLOGY ELECTRON MICROSCOPY IN-SITU HYBRIDIZATION AND THE POLYMERASE CHAIN REACTION'	1,2,4, 7-12, 14-17
Y	see the whole document	3,5,13, 18-21
Y	WO,A,91 12269 (MIKROGEN MOLEKULARBIOLOGISCHE ENTWICKLUNGS-GMBH) 22 August 1991 see the whole document	3,5,13, 17-21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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		AU-A-	7211591	03-09-91
		DE-D-	59101577	09-06-94
		EP-A-	0514413	25-11-92
		ES-T-	2052370	01-07-94
		JP-T-	5504143	01-07-93

WO-A-9104330	04-04-91	NL-A-	8902301	02-04-91
		EP-A-	0491824	01-07-92
